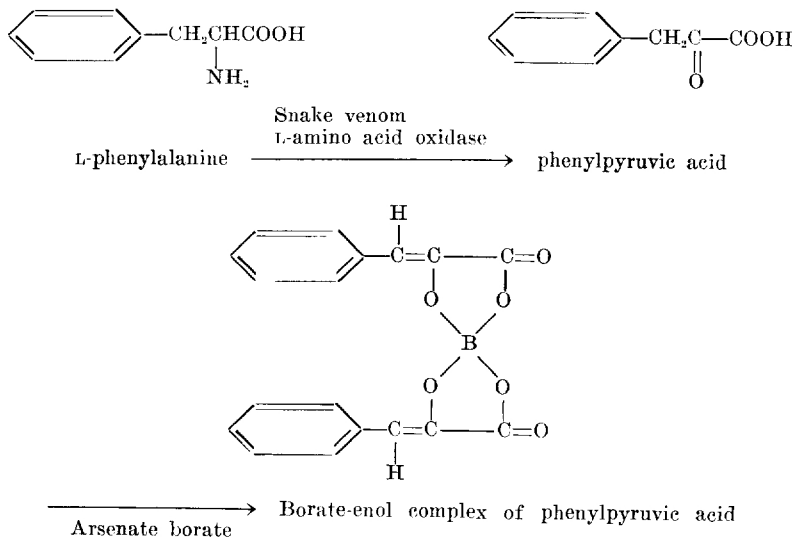


AN ENZYMIC SPECTROPHOTOMETRIC METHOD FOR THE
 DETERMINATION OF PHENYLALANINE IN BLOOD

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THERE is a need for a simple rapid method to measure phenylalanine in serum or plasma, not only as an aid in the diagnosis of phenylketonuria, but also in the evaluation of the effectiveness of a diet low in phenylalanine in the treatment of patients with this condition. The method most commonly used to measure phenylalanine in plasma is that of Udenfriend and Cooper,¹ in which plasma is deproteinized and treated with *Streptococcus faecalis* to decarboxylate L-phenylalanine to phenylethylamine. The latter is then determined colorimetrically after reaction with methyl orange. Although this method has been used successfully by many workers,²⁻⁶ it requires relatively large amounts of plasma and takes several hours to perform each set of analyses. Furthermore, extremely careful technique is necessary to avoid an appreciable and variable blank from methyl orange.

Another method for the determination of serum phenylalanine is that of Berry,⁷ by paper chromatography. This method, although simple, has limitations in quantitative analysis inherent in any method which depends upon estimation of the intensity of colored spots on paper chromatograms.



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A simple rapid method is described which permits the analysis of plasma L-phenylalanine in 0.1 ml. of serum without deproteinization. The method utilizes L-amino acid oxidase from snake venom to oxidize L-phenylalanine to phenylpyruvic acid. In the presence of arsenate and borate ions the resulting α -keto acid is rapidly converted to an enol-borate complex which has a high absorption in ultraviolet light. Catalase is added to protect the α -keto acid from peroxide formed in the oxidative deamination by L-amino acid oxidase.

Enol-borate complexes have been employed by Knox and Pitt⁸ to determine p-hydroxyphenylpyruvic acid oxidase activity in mammalian liver preparations. More recently these complexes have been used to measure the activity of several enzymes which either form or break down the aromatic α -keto acids related to phenylalanine, tyrosine, tryptophan, and histidine.⁹

METHOD

Reagents.—

1. Phosphate buffer: 0.2M sodium phosphate buffer, pH 6.5.
2. Arsenate-phosphate buffer: 2.0M sodium arsenate was dissolved in the 0.2M phosphate buffer, and the final pH was readjusted to 6.5 with dilute HCl.
3. Borate-arsenate reagent: 1.0M borate, dissolved in 2.0M arsenate, pH 6.5, (61.8 Gm. boric acid + 62.4 Gm. sodium arsenate [$\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$]) was adjusted to pH 6.5 with HCl and made up to 1 L.
4. Snake venom L-amino acid oxidase (the venom of *Crotalus adamanteus*^{*}): a suspension of dried venom in water was made which contained 10 mg. per milliliter. This was centrifuged, and the clear supernatant solution was removed and used. The enzyme solution is kept at 0 to 5° C. and remains active with little loss of activity for several days. The dry venom is stored in the refrigerator and maintains high enzyme activity for several months.

The activity of the L-amino acid oxidase preparation can be assayed by using 0.1 ml. of the standard phenylalanine solution in place of serum in the directions given below.

5. Catalase: crystalline beef liver catalase,† diluted 1:5 with 0.2M phosphate buffer, pH 6.5. The enzyme solution is kept at 0 to 5° C. and can be used for several weeks.
6. Standard phenylalanine solution: L-phenylalanine, 1 μ M per milliliter (0.1 ml. contains 16.5 μ g).
7. Blood serum: Blood is drawn and allowed to clot. (Some samples of heparinized plasma develop turbidity during analysis which makes them unsuitable for this determination.)

Procedure.—

Three 1.2 ml. quartz Beckman cuvettes with a 1 cm. light path are used for the determinations. The additions to each of the cuvettes are as follows:

	C ₁	E ₁	E ₂
PO ₄ buffer, 0.2M, pH 6.5‡:	0.39	0.39	0.39
Arsenate-phosphate, pH 6.5:	0.5	—	—
1M borate in 2M arsenate:	—	0.5	0.5
Catalase, 1:5 dilution:	0.01	0.01	0.01
Venom, 10 mg. per milliliter:	0.10	0.10	0.10
(Serum added later.)			

The contents are mixed and the duplicate experimental cuvettes, E₁ and E₂, are read against the control cuvette, C₁, at 308 m μ , in the Beckman spectrophotometer.§ If the addi-

*From the Ross Allen Reptile Institute, Silver Springs, Fla.

†From the Worthington Biochemical Corporation, Freehold, N. J.

‡If more than 0.1 ml. of serum is to be added, the volume of phosphate buffer must be reduced proportionately.

§Model DU with ultraviolet attachment.

tions have been made correctly, this reading should be approximately zero. Then 0.1 ml. of serum is added to cuvettes C₁, E₁, and E₂, mixed, and readings are taken at 1 or 2 minute intervals for 10 minutes at 308 mμ, to be certain that the enzyme activity is adequate, and that the reaction is essentially complete, as indicated by finding no further change in the optical density reading. Final readings are made at 308, 330, and 350 mμ at this time.

Calculations.—

Readings are taken at 330 and 350 mμ, as well as at 308 mμ, to correct for the absorption of the keto acids derived from tyrosine and tryptophan, since the latter 2 amino acids are also oxidized by the venom L-amino acid oxidase. The correction applied is based upon the relative absorption of the enol-borate complexes of these keto acids at 308, 330, and 350 mμ:

KETO ACIDS OF	RELATIVE OPTICAL DENSITY READINGS AT DIFFERENT WAVELENGTHS (mμ)		
	308	330	350
X = tyrosine	X	0.60X	0.06X
Y = phenylalanine	Y	0.10Y	0
Z = tryptophan	0.85Z	1.41Z	Z

Let the reading at 308 mμ = A; 330 mμ = B; 350 mμ = C

Then: $A = X + Y + 0.85Z$
 $B = 0.6X + 0.1Y + 1.41Z$
 $C = 0.06X + Z$

Solving these equations gives:

$X = 2.38B - 0.238A - 3.16C$ (1)
 $Y = 1.23A - 2.26B + 2.15C$ (2)
 $Z = 1.19C - 0.14B + 0.014A = C - 0.06X$ (3)

The absorption at 308 mμ caused by phenylpyruvic acid is given by solving for Y in equation (2):

$\frac{Y}{0.031} \times 10 = \mu\text{g phenylalanine per milliliter of serum (if 0.1 ml. of serum were analyzed). One microgram phenylalanine under these conditions of 1.1 ml., etc., at 308 m}\mu \text{ reads 0.031 O.D. units.}$

By the same means, solving for X in equation (1) and dividing: $\frac{X}{0.045} \times 10 = \mu\text{g tyrosine per milliliter of plasma (1 }\mu\text{g tyrosine under these conditions reads 0.045 at 308 m}\mu\text{); and, similarly, using equation (3): } \frac{Z}{0.033} \times 10 = \mu\text{g tryptophan per milliliter of serum (1 }\mu\text{g of tryptophan reads 0.033 at 350 m}\mu\text{).}$

RESULTS AND DISCUSSION

Specificity.—The L-amino acid oxidase of snake venom catalyzes the oxidation of a number of amino acids. However, only 4 of these are present in appreciable quantities in serum which yields α-keto acids with highly absorbing enol-borate complexes; these are phenylalanine, tyrosine, tryptophan, and histidine. Fortunately, histidine is not oxidized under the experimental conditions described above, and the relative contribution of each of the other 3 amino acids can easily be determined from the absorption of the combined complexes at 3 different wavelengths. Therefore, this method permits the simultaneous determination of phenylalanine, tyrosine, and tryptophan in serum. If the value of phenylalanine alone is desired, the correction for absorption caused by the keto acids of tyrosine and tryptophan would appear to be a disadvantage of the method. This is partly compensated for by the commercial availability of the

enzyme, its stability, and its high activity. Furthermore, the ease with which both phenylalanine and tyrosine can be measured is of special interest in some studies on phenylketonuria; for example, in the detection of the heterozygous carrier of this trait by the ratio between the levels of phenylalanine and tyrosine in the blood after the administration of phenylalanine as a tolerance test.⁵

The wide range of substrates which can be used with this enzyme has made it possible to extend this method to measure a number of other aromatic amino acid analogues and antimetabolites, such as p-fluorophenylalanine, m-tyrosine, monoiodotyrosine, mononitrotyrosine, and β -2-thienylalanine. These compounds are also rapidly oxidized by the L-amino acid oxidase of snake venom, and the resulting keto acid products can be measured as enol-borate complexes.¹⁰

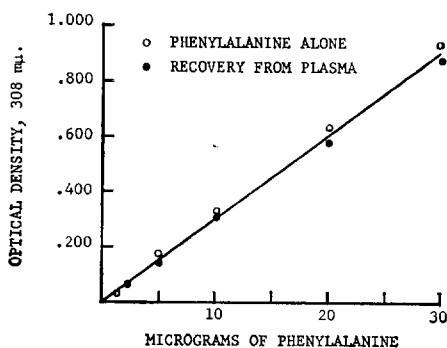


Fig. 1.—Graph of the optical density at 308 $m\mu$ versus concentration of phenylalanine when determined in standard solutions and when added to normal plasma. The linearity between optical density and phenylalanine concentration is demonstrated.

Reproducibility and Accuracy.—The method described is well suited to measuring the elevated blood level of phenylalanine found in untreated phenylketonuric individuals and to following the effectiveness of a diet low in phenylalanine in lowering the serum level in these individuals. The specificity of the absorption spectrum of the phenylpyruvic acid enol-borate complex and the ease and rapidity of the analysis permit a large number of determinations to be carried out within a short period of time. Fig. 1 demonstrates the linear relationship between phenylalanine concentration and optical density change between 2 and 30 μg analyzed alone and the recovery of phenylalanine added to normal plasma in this range. These results are in agreement with the theoretic values expected. However, the precision of the analytic method is lower in analyses of phenylalanine in normal serum than when the values are elevated as in serum of an individual with phenylketonuria. In normal serum the contributions of tyrosine and tryptophan to the reading at 308 $m\mu$ are appreciable. Even though duplicate pairs of analyses in a series of normal serum samples analyzed on successive days rarely disagreed by more than 7 per cent, larger aliquots of serum would be desirable if the method were to be used to measure, with high accuracy, the endogenous phenylalanine, tyrosine, and tryptophan levels. Attempts to carry out the analyses directly on 0.2 or 0.3 ml. of serum have been only partly successful, since a turbidity often occurs. For this reason,

several methods were tested to deproteinize the sample before analysis. Preliminary experiments have been made using perchloric acid precipitation followed by neutralization and the removal of $KClO_4$, as described by Segal, Blair, and Wyngaarden,¹¹ before enzymatic assay of blood pyruvate. This procedure for deproteinization appears to be satisfactory in eliminating the turbidity problem, and the solution which results can be used directly in the enzymatic assay.

Phenylalanine Levels in Normal and Phenylketonuric Individuals.—The serum phenylalanine levels of 30 adult blood bank donors in a nonfasting condition were found to have an average value of 1.55 mg. per cent (range, 0.84 to 2.64 mg. per cent). It is probable that the level in normal subjects in a fasting condition is somewhat lower than these values. In a smaller number of analyses in laboratory workers in a fasting condition, the group average is slightly lower than the above value.

The level of phenylalanine in a group of 10 phenylketonuric individuals on a regular diet was found to have an average value of 36.8 mg. per cent (range, 26.2 to 59.1 mg. per cent). These values in normal and in phenylketonuric people are in good agreement with those in the literature^{7, 12-14} obtained by other methods. The wide difference in the levels of normal and phenylketonuric individuals makes the analysis of serum phenylalanine a valuable diagnostic procedure.

SUMMARY

A simple enzymatic spectrophotometric method has been described for the quantitative determination of phenylalanine in serum. The method is based upon the measurement of the absorption of the enol-borate complex of phenylpyruvic acid generated enzymatically from phenylalanine by L-amino acid oxidase of snake venom. For elevated serum levels of phenylalanine the method is rapid, specific, and precise. It should be useful as a confirmatory test in the diagnosis of suspected phenylketonuria and in the evaluation of the effectiveness of a diet low in phenylalanine. The values obtained by this method agree well with those in the literature obtained by other techniques. Tyrosine and tryptophan are also determined by this method, and a suitable modification of the method is described which should allow an accurate estimation of these amino acids and phenylalanine in normal serum.

REFERENCES

1. Udenfriend, S., and Cooper, J. R.: Assay of L-Phenylalanine as Phenylethylamine After Enzymatic Decarboxylation; Application to Isotopic Studies, *J. Biol. Chem.* **203**: 953, 1953.
2. Meister, A., Udenfriend, S., and Bessman, S. P.: Diminished Phenylketonuria in Phenylpyruvic Oligophrenia After Administration of L-Glutamine, L-Glutamate or L-Asparagine, *J. Clin. Invest.* **35**: 619, 1956.
3. Hsia, D. Y.-Y., Driscoll, K. W., Troll, W., and Knox, W. E.: Detection by Phenylalanine Tolerance Tests of Heterozygous Carriers of Phenylketonuria, *Nature, London* **178**: 1239, 1956.
4. Hsia, D. Y.-Y., and Paine, R. S.: Phenylketonuria: Detection of the Heterozygous Carrier, *J. Ment. Deficiency Res.* **1**: 53, 1957.
5. Hsia, D. Y.-Y.: Phenylketonuria: The Phenylalanine-Tyrosine Ratio in the Detection of the Heterozygous Carrier, *J. Ment. Deficiency Res.* **2**: 8, 1958.

6. Knox, W. E., and Messinger, E. C.: The Detection in the Heterozygote of the Metabolic Effect of the Recessive Gene for Phenylketonuria, *Am. J. Human Genet.* **10**: 53, 1958.
7. Berry, H. K.: Paper Chromatographic Method for Estimation of Phenylalanine, *Proc. Soc. Exper. Biol. & Med.* **95**: 71, 1957.
8. Knox, W. E., and Pitt, B. M.: Enzymatic Catalysis of the Keto-Enol Tautomerization of Phenylpyruvic Acids, *J. Biol. Chem.* **225**: 675, 1957.
9. Lin, E. C. C., Pitt, B. M., Civen, M., and Knox, W. E.: The Assay of Aromatic Amino Acid Transaminations and Keto Acid Oxidation by the Enol Borate-Tautomerase Method, *J. Biol. Chem.* **233**: 668, 1958.
10. La Du, B. N., and Michael, P. J.: A New Assay for Analogues and Antimetabolites of Tyrosine and Phenylalanine. In preparation.
11. Segal, S., Blair, A. E., and Wyngaarden, J. B.: An Enzymatic Spectrophotometric Method for the Determination of Pyruvic Acid in Blood, *J. LAB. & CLIN. MED.* **48**: 137, 1956.
12. Hsia, D. Y.-Y., and Driscoll, K. W.: Detection of the Heterozygous Carriers of Phenylketonuria, *Lancet* **2**: 1337, 1956.
13. Jervis, G. A., Block, R. J., Bolling, D., and Kanze, E.: Chemical and Metabolic Studies on Phenylalanine. II. The Phenylalanine Content of the Blood and Spinal Fluid in Phenylpyruvic Oligophrenia, *J. Biol. Chem.* **134**: 105, 1940.
14. Bickel, H., Gerrard, J., and Hickmans, E. M.: The Influence of Phenylalanine Intake on the Chemistry and Behavior of a Phenylketonuric Child, *Acta paediat.* **43**: 64, 1954.